

Isolation and Translation of Plant Messenger RNA¹

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ELAINE M. TOBIN² AND ATTILA O. KLEIN

Department of Biology, Brandeis University, Waltham, Massachusetts 02154

ABSTRACT

A fraction of the RNA species isolated from *Lemna gibba* G-3 consists of molecules with attached sequences of polyadenylic acid. This polyadenylic acid-containing fraction, separated from total RNA by adsorption onto oligothymidylic acid-cellulose, was shown to be mRNA by its ability to serve as template in a cell-free translation system derived from wheat germ. The products of translation were characterized by electrophoresis. This method permitted the comparison of mRNA from plants grown under different light conditions. Such plants were shown to possess qualitative and quantitative differences in their mRNA complements.

Many attempts have been made to discover the level at which specific developmental changes are controlled in plants. Some of these attempts have focused on the possibility of transcriptional control (5, 8, 9, 14-16, 18, 19, 21, 24, 32, 36). Until recently, attempts to isolate and study mRNA from plants depended on the rapid labeling of certain fractions which were not fully characterized (12, 16, 20). With the discovery that most eucaryotic mRNA contains poly(A)³ sequences, a simple procedure for mRNA isolation has become available. Poly(A) RNA has been found now in a number of plant species (10, 13, 23, 30, 33-35) as well as in other eucaryotic organisms (7).

We have undertaken to isolate poly(A) RNA from *Lemna gibba* G-3 and to use a heterologous cell-free translation system to show that this poly(A) RNA is indeed mRNA. In addition, we have tried to develop a general method for examining differences in mRNA populations in plants growing under different conditions. By characterizing the translation products of mRNA one can show differences in the relative content of different messengers as well as in the over-all level of mRNA.

Putting duckweed into the dark changes its growth pattern (25, 29). We have chosen initially to compare mRNA from *Lemna* placed in the dark with the mRNA content after returning the plants to the light. It should now also be possible to make comparisons between the mRNA complement of plants in various developmental stages.

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² Present address: Department of Biology, University of California, Los Angeles, Calif. 90024.

³ Abbreviations: poly(A): polyadenylic acid; poly(A) RNA: RNA containing a sequence of polyadenylic acid; oligo (dT) cellulose: oligo-thymidylic acid covalently linked to cellulose.

MATERIALS AND METHODS

Lemna gibba G-3 were grown aseptically on E medium (6) in constant light at 22 C. Plants were harvested on Miracloth (Calbiochem), rinsed with distilled H₂O, and frozen in liquid nitrogen. If the tissue was not used immediately, it was stored at -20 C.

Tissue was ground in a Waring blender in two volumes (v/w) of 0.1 M sodium acetate, pH 5.2, containing 0.2 M LiCl, 1% SDS, and 0.5% diethylpyrocabonate (Baycovin, Naftone, Inc., N.Y.) for 2 min at top speed. After adding isoamyl alcohol (1-2%) to control foaming, the homogenate was extracted with 2 volumes of hot phenol (60 C) containing 1% 8-hydroxyquinoline and saturated with the grinding buffer. The phenol phase was extracted with 0.5 volume of the grinding buffer, and the combined aqueous phases were extracted two more times with phenol. Nucleic acids were precipitated overnight with 2 volumes of cold ethanol, dissolved in 0.01 M tris, pH 7.4, and reprecipitated in the presence of 0.1 M sodium acetate with ethanol.

When RNA uncontaminated by DNA was required, the nucleic acid precipitate was dissolved in 0.01 M MgCl₂, 0.01 M tris, pH 7.4, and was treated with ribonuclease-free DNase (25 µg/ml [Worthington]) for 1 hr at 37 C. The solution was then brought to 0.01 M EDTA, 0.2 M LiCl, and 0.5% SDS, extracted twice with phenol, and the RNA was precipitated with ethanol. The precipitate was dissolved and reprecipitated in the presence of 4 M potassium acetate.

The RNA was further purified in all cases by extraction with 2-methoxyethanol and precipitation with cetyltrimethylammonium bromide, according to the procedure of Bellamy and Ralph (3).

Poly(A) containing RNA was isolated on oligo (dT) cellulose columns (2). Total RNA was dissolved in 0.5 M salt (KCl or NaCl) in 0.01 M tris, pH 7.5, and applied to a column containing 0.2 to 0.25 g of oligo (dT) cellulose (T-1, Collaborative Research, Waltham, Mass.). Poly(A) RNA binds to the oligo (dT) cellulose under this high salt condition, but the bulk of the RNA does not bind. The sample was washed onto the column with additional high salt buffer until the eluate did not contain any RNA. The column was then washed with 0.1 M salt, 0.01 M tris, pH 7.5, and the poly(A) RNA was eluted with 0.01 M tris, pH 7.5. The column buffers were made either with KCl or with NaCl and 0.5% SDS. Elution of some poly(A) RNA occurred from some oligo (dT) batches at 0.1 M salt. In some experiments washing with 0.1 M salt was omitted, and the poly(A) RNA was eluted after washing with the original 0.5 M salt buffer. The RNA in fractions from the column was precipitated with ethanol and recovered by centrifugation.

In order to demonstrate the existence of poly(A) sequences, RNA was digested with T-1 and pancreatic ribonucleases, which leave poly(A) sequences intact, according to the method of Lee *et al.* (22). After phenol extraction, the poly(A) segment was isolated on oligo (dT) cellulose columns, and its base composition was determined.

TABLE I. Base Composition as Percentage of Total

Sample	C	A	G	U
Total RNA	25	23	30	22
Unbound RNA	25	22	30	23
RNA eluted with 0.1M KCl	23	31	26	20
After RNase treatment	<0.5	>95	<2	<2

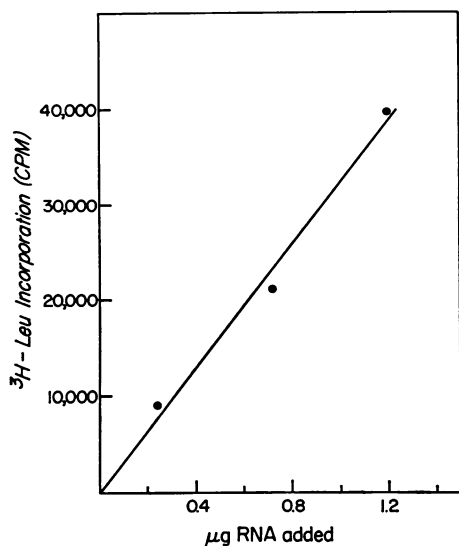


FIG. 1. Wheat germ cell-free translation system: dependence of ^3H -leu incorporation on added poly(A) RNA. ^3H -leu incorporation into trichloroacetic acid-precipitable fraction of the 50- μl reaction mixture. The endogenous background incorporation (with no added RNA) has been subtracted.

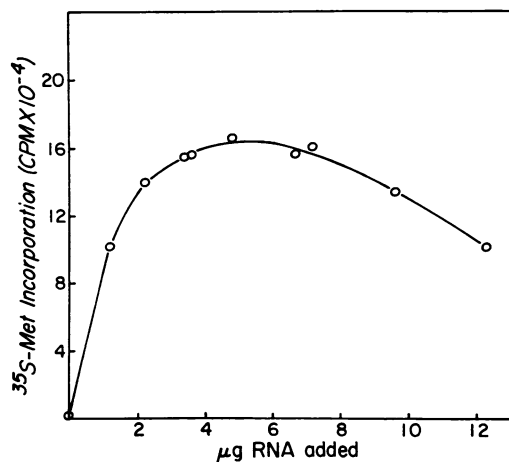


FIG. 2. Wheat germ cell-free translation system: saturation and inhibition of ^{35}S -met incorporation by added poly(A) RNA. RNA which did not bind to the oligo (dT) cellulose column resulted in incorporation of 1.2 to 2.2×10^4 cpm when added in concentrations ranging from 1.1 to $8.6 \mu\text{g}/50 \mu\text{l}$. Poly(A) RNA stimulated the incorporation up to almost 80 times the level of the background.

To determine base ratios of the various fractions of RNA, carrier-free $^{32}\text{PO}_4^{3-}$ was added to growing cultures of *Lemna* for 18 hr before extraction of the RNA. The RNA fractions from the oligo (dT) cellulose columns were desalted on Sephadex G-10 columns, lyophilized, and hydrolyzed in 0.2 M NaOH at 37°C for

18 hr. The bases were separated by high-voltage paper electrophoresis (31). The radioactive areas corresponding to the four nucleotides were located by autoradiography. The spots were cut out and their radioactivity was determined with a scintillation counter.

Poly(A) containing RNA was translated in an *in vitro* protein synthesizing system derived from wheat germ (28). Either ^3H -leucine or ^{35}S -methionine (New England Nuclear) was used to label the translation products. To determine the extent of translation, aliquots of the 50- μl reaction mixture were treated with 10% trichloroacetic acid and the radioactivity incorporated into

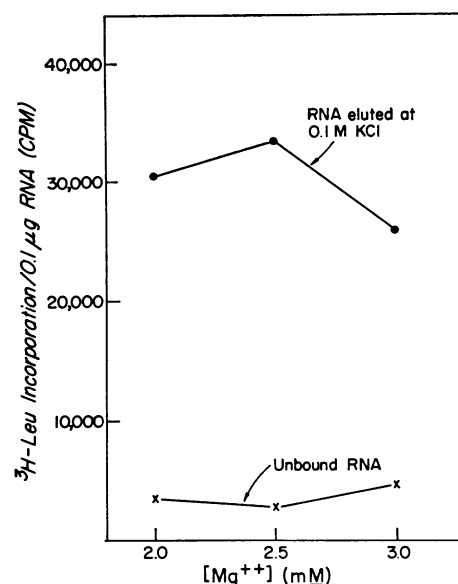


FIG. 3. Wheat germ cell-free translation system: Mg^{2+} dependence with added *Lemna* mRNA. ^3H -leu incorporation in the 50 μl of reaction mixture/ $0.1 \mu\text{g}$ of added RNA is plotted after subtracting the endogenous background. RNA which did not bind to oligo (dT) cellulose stimulated incorporation only slightly.

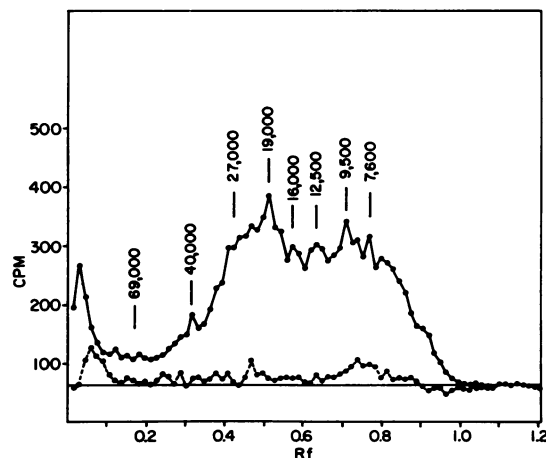


FIG. 4. Electrophoresis of ^3H -leu labeled translation products on acrylamide-SDS gels. RNA was isolated from *Lemna* grown in constant light. The translation products were subjected to electrophoresis on cylindrical gels. The gels were sliced into 1-mm segments. The top curve shows the radioactive products from poly(A) RNA, and the bottom curve shows the radioactive products from that RNA which did not bind to oligo (dT) cellulose. The straight line is the scintillation counter background. The mol wt were estimated from a standard curve derived from proteins of known mol/wt.

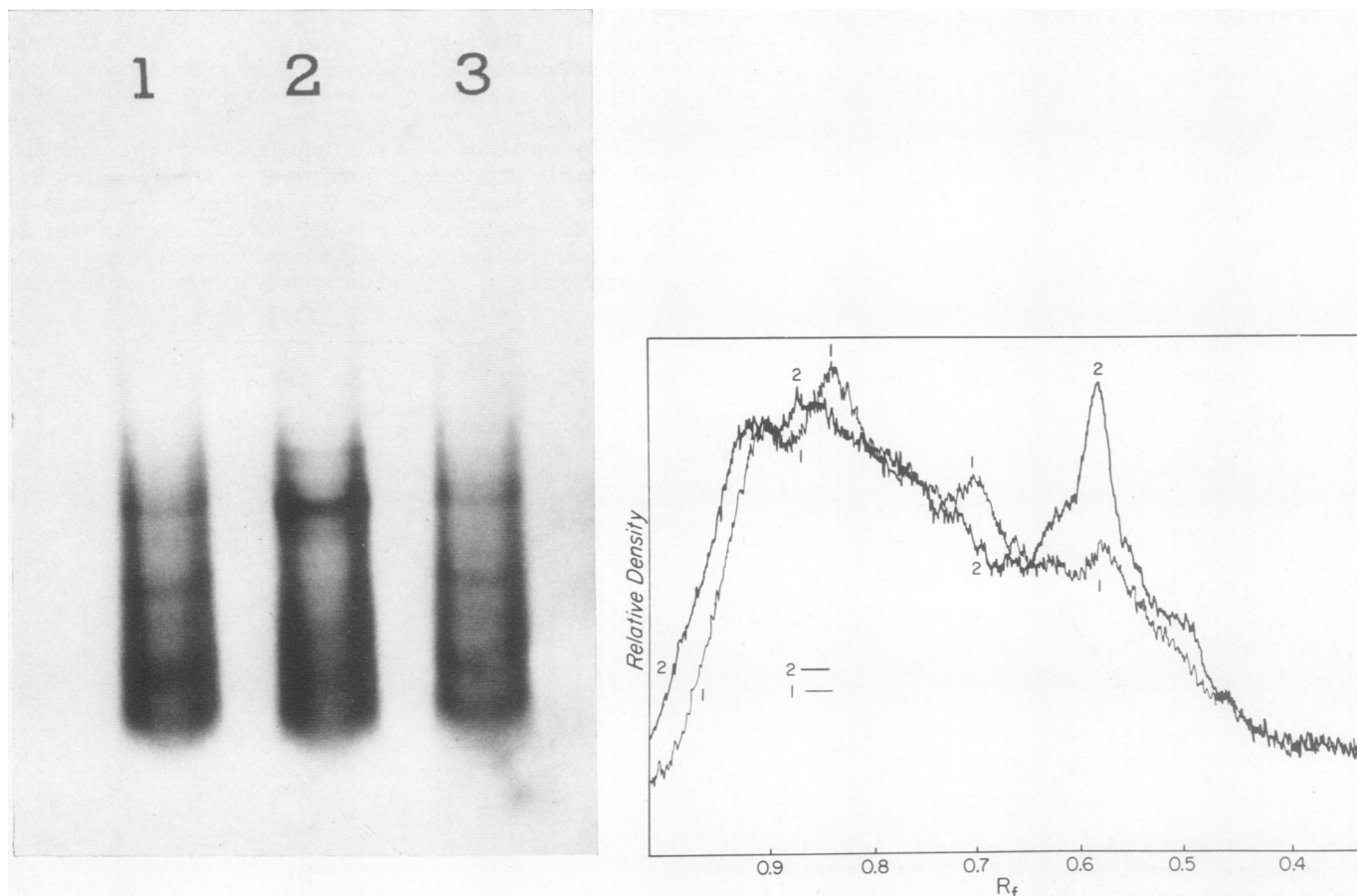


FIG. 5. Comparison of ^{35}S -met-labeled translation products of mRNA isolated from *Lemna* under two different conditions of growth. Fluorography of the dried slab gel. 1 and 3: products of replicate translations of mRNA from *Lemna* that received 24 hr of light after 59 hr of darkness. 2: products of translation of mRNA from *Lemna* that were in darkness for 59 hr.

FIG. 6. Densitometric tracing of the fluorogram in Figure 5. Curves 1 and 2 correspond to banding patterns 1 and 2 in Figure 5.

the washed precipitate was determined. The translation products were characterized by electrophoresis on 10% polyacrylamide-0.27% methylene bisacrylamide gels containing 0.1% SDS. The products were precipitated from the remainder of the reaction mixture with cold 10% trichloroacetic acid containing 3% casamino acids (Difco), washed 3 to 4 times with the same solution, dissolved in 6 M urea, 1% SDS, 1% mercaptoethanol, 0.02 M tris, pH 8.4, and placed for 1 to 5 min in a boiling water bath. The electrophoresis buffer was 0.1% SDS, 0.075 M NaPO_4 , pH 7.2. Both cylindrical ($d = 6$ mm) and slab ($150 \times 200 \times 1.5$ mm) gels were used. After electrophoresis, cylindrical gels were sliced into 1-mm segments, dissolved in toluene containing 6% Protosol (New England Nuclear) and 0.04% Omnifluor (New England Nuclear), and the radioactivity was measured. Relative radioactivity of bands in the slab gels was determined by the fluorographic method of Bonner and Laskey (4). The density profiles of the bands on the x-ray film were recorded with a Joyce-Loebl microdensitometer.

Molecular weight estimates of the radioactive bands on gels were made by including proteins of known mol wt in parallel with the samples during electrophoresis.

In all procedures except the electrophoresis, all glassware was acid washed or autoclaved, and all solutions were autoclaved unless they contained alcohol or SDS.

RESULTS

RNA from *Lemna* was found to include a fraction which contains poly(A) sequences. This fraction was isolated on oligo (dT) cellulose columns. Table I shows that the RNA binding to these

columns is indeed enriched in adenylic acid. It contains sequences resistant to pancreatic and T-1 ribonucleases; these sequences contain more than 95% adenylic acid.

Usually about 1% of the total A_{260} applied to the columns bound to the oligo (dT) cellulose. This amount varied somewhat with different batches of oligo (dT) cellulose, and ranged from 0.3% to 1.5%. The average from 14 preparations was 0.9%.

Perry *et al.* (26) reported that loss of poly(A) sequences is reduced if extractions are done with phenol plus chloroform rather than with phenol alone at room temperature and a near neutral pH buffer. However, under the extraction conditions used here (low pH and 60°C initial extraction), the amount of poly(A) RNA recovered was not affected by substituting chloroform-phenol (1:1) for the phenol.

The poly(A) RNA was shown to be messenger RNA by translating it in a heterologous cell-free system derived from wheat germ (28). This system has a low background level of endogenous activity and has been shown to translate accurately tobacco mosaic virus coat protein and rabbit hemoglobin messenger RNAs. Figure 1 shows that the amount of radioactivity incorporated into the trichloroacetic acid precipitable products is directly proportional to the amount of poly(A) RNA added to the translation mixture. At higher RNA concentrations, incorporation becomes nonlinear, and beyond about 5 μg of RNA/50 μl of reaction mixture incorporation is actually inhibited (Fig. 2). The Mg^{2+} optimum for the *Lemna* mRNA translation is at 2.5 mM Mg^{2+} (Fig. 3) and the K^+ optimum is at 80 mM K^+ . RNA which does not bind to oligo (dT) cellulose gives very little stimulation of amino acid incorporation in the wheat germ system (Fig. 3).

Translation products from mRNA isolated from *Lemna* grown

in constant light were characterized by electrophoresis on cylindrical polyacrylamide-SDS gels. The pattern of radioactivity of the peptides is shown in Figure 4. The major portion of the peptides had mol wt in the range 5,000 to 40,000 daltons.

Analysis of the products of the cell-free translation allows comparison of mRNA populations under different conditions of growth. We made such a comparison between the poly(A) RNA complement derived from *Lemna* at two different points in a sequence of alternating light-dark conditions. Light-grown *Lemna* were transferred to darkness for 59 hr then returned to the light for 24 hr. Half of the flasks were harvested at the end of the dark period and the rest were harvested at the end of the additional 24 hr of light. Poly(A) RNA was isolated simultaneously from both samples, translated in the wheat germ system, and the trichloroacetic acid-precipitable products were analyzed by electrophoresis on slab gels, followed by fluorography and densitometry. Figure 5 shows the radioactive bands produced on the x-ray film. At least eight separate bands can be seen after this 29 hr exposure. Replicate translations give identical patterns of bands. The samples applied to gels 1 and 3 were from two separate translations of mRNA from the plants that received light after the dark treatment, whereas the sample applied to gel 2 was translated from the mRNA from the plants harvested at the end of the dark treatment. Figure 6 shows the densitometric tracing of the relative intensities of the bands on gels 1 and 2. Each gel had approximately equal amounts of radioactivity applied to them. It can be easily seen that the relative amount of mRNA for a number of proteins varies considerably between the two preparations.

DISCUSSION

Jacobson and Zwar (13) have recently shown that poly(A) RNA is increased relative to other species of RNA when barley aleurone tissue is treated with gibberellic acid for 16 hr. They suggest that this represents a specific increase in the synthesis of mRNA. Ho and Varner (11) have also found an increased synthesis of poly(A) RNA in gibberellin treated barley aleurone layers. The translation of the isolated RNA provides more certain evidence that this RNA is indeed mRNA. This evidence is better than can be obtained by measuring incorporation of labeled adenosine into poly(A) RNA. By characterizing the translation products one can hope to look for more specific changes in mRNA than can be detected by simply measuring the poly(A) content of RNA. Verma *et al.* (34) have isolated poly(A) RNA from soybean root nodules and translated it in a cell-free system from wheat embryos. They were able to show that a large part of the translation products could be precipitated by antibody to the root nodule leg-hemoglobin. In the experiments presented here, we have also translated poly(A) RNA and have furthermore shown that it is possible to detect qualitative as well as quantitative changes in mRNA content by comparing the products of translation.

Because not all mRNA contains poly(A) (1), the methods used here would not be able to detect changes in such nonpoly(A) mRNA. Although these experiments do not eliminate the possibility that the translated mRNA is a fragment of the native messenger, such a possibility seems unlikely. The methods used select for both an intact 3'OH end (the end to which the long poly(A) sequence is attached in all eucaryotic mRNAs so far examined) and an intact initiation site at the 5'OH end.

Rhoads *et al.* (27) have shown that at least in the rabbit reticulocyte lysate protein-synthesizing system, no translation of mRNA fragments could be detected in partially degraded preparations. It is possible that translation of the mRNA may be incomplete, resulting in the appearance of shorter than expected proteins. Because the mol wt range of the products is lower than the range of the mol wt of the proteins found *in vivo* (E. Tobin, unpublished), this may indeed be the case. However, because the electrophoretic separation pattern of the translation products contains numerous discrete bands, and because separate transla-

tions of the same mRNA produce products with identical banding patterns (Fig. 5), either many proteins must be completely translated, or their translation is consistently aborted at the same point. Complete translation of hemoglobin (28) and preproparathyroid hormone (17) by the wheat-germ cell-free system have been reported. Even if not all of our mRNA is completely translated, it should still be possible to look for differences in two mRNA populations by our methods.

In this report we have been able to show one such difference. In *Lemna gibba* light and darkness do affect the mRNA population; when the *Lemna* were transferred back to light after two days of darkness, some messenger species decreased while others increased. The largest difference found here was nearly 2-fold in a polypeptide peak that corresponds to a mol wt of about 18,000. The actual difference is probably even more dramatic, because the electrophoretic separation of the peptides is not complete.

The next step in this work might be to try to positively identify one or more of the polypeptide products by immunological methods and to try to relate changes in the levels of their mRNA to changes in the protein production *in vivo*.

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